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13. ABSTRACT *(Maximum 200* It is now well documented that angiogenesis is required for the growth of both primary and metastatic breast cancer. Hence, inhibition of tumor-associated blood vessel growth should be an effective means of treating this disease. Our laboratory has investigated the structure/function relationships of a potent angiogenic mediator, termed angiogenin (Ang). Several inhibitors of its functions have been produced by us and shown previously to possess antitumor activity in preclinical models against a range of human solid tumor types. On this basis we were awarded a grant from the Department of Defense to extend our studies to the treatment of breast cancer. During the second year of funding we have demonstrated that both a monoclonal antibody (mAb 26-2F) and the Ang antisense agent JF2S potently inhibit the establishment of primary tumors following the subcutaneous injection of breast cancer tumor cells into athymic mice. In the case of JF2S this inhibition is complete. We have also developed an orthotopic metastatic model for use in therapy trials. Using this assay system we have for the first time demonstrated that Ang antagonists dramatically interfere with the formation of lung metastases arising from implantation of breast cancer cells into the mammary fat pad. Thus, we continue to validate our contention that targeting Ang should be a novel means of combating metastatic carcinoma of the breast.

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James W. Fett 9/21/98

PI - Signature Date

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INTRODUCTION

Background. Carcinoma of the breast is now the most commonly diagnosed cancer among women in the United States and is second only to lung cancer in female cancer-related deaths. It is estimated that over 178,000 new cases will be diagnosed and approximately 44,000 individuals will die from this disease during 1998 (1). While local noninvasive breast cancer in many cases is curable, a sizeable proportion of these patients experience relapse. Furthermore, metastatic disease to distant sites is at present generally considered incurable with treatment aimed at increasing survival while maintaining a reasonable quality of life. Thus, more effective therapeutic maneuvers for the treatment or prevention of advanced, metastatic breast cancer are urgently needed.

Ideally, therapeutic goals for those patients with advanced breast cancer include prevention of development of metastasis where it has not occurred and eradicating occult or detectable metastatic lesions already present - both in conjunction with eliminating the primary cancer. Alternatively, in cases where complete cure or prevention of metastasis is not possible, managing patients in a state of metastatic "stasis" would also be a desirable outcome. We, as others, believe that novel therapeutic strategies that include an *antiangiogenic* component offer realistic hope of achieving these goals in the near future.

The critical contribution of angiogenesis - the development of a hemovascular network - to the growth of solid tumors is now undisputed (2). Angiogenesis also is a prerequisite for the development of metastasis since it provides the means whereby tumor cells disseminate from the original primary tumor, traverse through the circulation, and establish at distant sites (reviewed in ref. 3). Therefore, interference with the process of tumor-induced angiogenesis should be an effective therapy for both primary and metastatic cancers. Our own work along with the recent reports that drugs (4) as well as antibodies to a secreted angiogenesis factor (5) suppress the growth and metastatic spread of tumors in animals now lend solid experimental support to this proposition. Indeed, at least nine antiangiogenic agents are in clinical trials for treatment of several angiogenesis-dependent diseases, including cancer (M. J. Folkman, personal communication).

Importantly, the degree of angiogenesis in early-stage breast cancer correlates positively with metastatic recurrence and survival (6). Hence, interference with the angiogenic process in breast cancer should have profound therapeutic consequences.

Angiogenin. The recognized importance of neovascularization in cancer has prompted intensive efforts toward identifying the chemical mediators involved in this process. We have since 1985 studied the structure/function relationships of a potent tumor-associated mediator of the angiogenic process that we named angiogenin (Ang) (7). It has subsequently been extensively characterized both structurally and functionally. The 3-dimensional structure of Ang is now known (8) and is providing a basis for rational design of potential therapeutic inhibitors. Several antagonists of Ang's activity are available to us and are being evaluated as potential cancer therapeutics. Since when we initiated these studies in the mid-1980's reproducible models for the study of human tumor metastasis in mice were not available, we developed a subcutaneous (sc) tumor model in athymic mice which mimics metastasis to investigate the effect of Ang antagonists on the establishment and growth of human tumor xenografts. This model (termed sc prophylactic), in which a small number of tumor cells are injected, approximates in its early stage metastatic disease where a limited number of tumor cells "seed" to a site and establish and grow only if, among other criteria, they receive an adequate supply of blood vessels from the host. In initial studies we demonstrated that prophylactic treatment with the neutralizing antiAng monoclonal antibody (mAb) 26-2F (the antibody is described in ref. 9), which is not cytotoxic to tumor cells *in vitro*, in all cases delayed the appearance of HT-29 colon adenocarcinoma tumors and, strikingly, completely

prevented their establishment in 25% of the mice (10). In subsequent studies adjustment of dosages and duration of administered antagonists enabled us to prevent completely the appearance of tumors in up to 60% of the mice using either of two mAbs or an Ang-binding protein with no observable adverse effects on the animals (11). Importantly, histological examination by our collaborator Dr. Marc E. Key, Vice President of Dako Corp., revealed a statistically significant decrease in the vascular density of those tumors that did develop in the Ang antagonist-treated mice. Thus, the availability of functional Ang is critical for the establishment of these tumors suggesting a therapeutically useful approach to the treatment of Ang-dependent malignancy. We have to date demonstrated that Ang antagonists are effective in inhibiting the growth of human colon, prostate, lung, brain, fibroblast and melanocyte tumors in preclinical mouse models.

Thus, with this extensive background and experience we were well positioned to extend our antiAng therapeutic strategies to the study of human breast carcinoma. A report of our most recent work supported by the Department of Defense on breast cancer follows.

BODY

I. Summary of Year 1 Progress (Sept. 1, 1996 - Sept. 1, 1997)

During the first year of support, as detailed in our first Progress Report, goals were met as follows according to the Statement of Work:

Specific Aim 1: Antitumor effects of individual Ang antagonists

- Orthotopic mouse models for *primary* growth in the mammary fat pad (mfp) of both the MDA-MB-435 cells (estrogen-independent) and MCF-7 cells (estrogen-dependent) cells were utilized to show that an Ang neutralizing murine monoclonal antibody, mAb 26-2F, potently inhibited the establishment of both of these cell types in this setting.
- Experiments to standardize a model to evaluate effects of Ang antagonists on the growth of *metastasis* resulting from orthotopic mfp injection of MDA-MB-435 cells were initiated. Following published protocols we performed three experiments but were disappointed with the number of control animals which developed metastatic lesions in the regional lymph nodes and lungs. Therefore, we contacted the expert in this field, Dr. Janet E. Price of the M. D. Anderson Cancer Center, who advised us to examine the additional parameters of removing the primary tumor surgically at 12 weeks and increasing the time to sacrifice to at least 17 weeks. These modifications in protocol were begun and experiments underway at the end of Year 1.
- Although not a part of the original research plan, we reported that an antisense oligonucleotide phosphorothioate, designated JF2S, was capable of inhibiting the synthesis of Ang in breast cancer cells *in vitro* and the subsequent growth of these treated cells after injection into the mfp of nude mice. As a result, we incorporated this promising class of drugs into our experimental protocols and demonstrated that JF2S was as potent as mAb 26-2F in interfering with the establishment of primary tumors derived from both MDA-MB-435 and MCF-7 cells. Antisense therapeutic strategies have now been incorporated into our research program. [Note: A revised Statement of Work reflecting this new direction is included in this report on pages 13-15].
- Drug development. As part of Aim 1 lead antiAng compounds are to be developed for clinical use and, in addition, small molecule drugs are to be prepared for inclusion during Years 3 & 4 into our experimental protocols. Although planned for Year 2, a chimeric antibody, designated cAb 26-2F, was constructed during the first year of funding and shown to be as effective as its murine counterpart, mAb 26-2F, with regard to its anti-tumor properties. A report on this work has now been published (see Appendix). Humanization of this antibody is ongoing. Two lead

small molecule drugs were also identified based on their capacity to inhibit the enzyme activity (necessary for biological activity) of Ang. At the end of Year 1 molecular modeling studies were in progress using both NMR and x-ray crystallography to build compounds of the necessary binding strength required for therapeutic testing in our model systems.

- Immunohistochemical analysis of human breast carcinoma specimens revealed, in all cases, strong staining for Ang protein. This finding indicates that Ang should indeed be a valid molecular target for the treatment of breast cancer clinically.

II. Progress Report: Year 2 (Sept. 1, 1997 - Sept. 1, 1998)

Specific Aim 1: Antitumor effects of individual Ang antagonists

Preclinical Mouse Model (sc prophylactic) for Primary Breast Cancer Growth. In the MDA-MB-435 model used in Year 1, 50 x 10^4 cells/mouse were mixed with the Ang antagonist and injected sc into the surgically exposed mfp of athymic mice. Follow-up injections of Ang antagonist were then given locally for 28 days. Using this model we were able to delay, and in a subset of mice (~40%), completely prevent the establishment of tumors using both mAb 26-2F and antisense JF2S. However, upon cessation of treatment we observed that tumors subsequently developed in several animals. In previous work using a similar model for prostate cancer we had found that reducing the number of tumor cells injected to only that needed to produce tumors in 100% of control mice resulted in a screening assay that was more predictive of efficacy in a separate model of metastasis. We thus were able to mimic in the sc screening assay a metastatic situation. (We have described this general process in ref.11). Therefore, for the present study a series of experiments were performed in which the number of injected breast cancer cells was progressively lowered concomitant with increasing the duration of treatment until day 49. As shown in Table 1, all control PBS-treated mice developed tumors following injection of either 12.5-, 6.25- or 4 x 10^4 cells/mouse. In contrast, at a dose of 2 x 10^4 cells/mouse no tumors developed in several of the control in two separate experiments. Therefore, this dose of initial cell inoculum was deemed too low and has been discontinued from consideration. We also observed, in two separate experiments, that decreasing the cell dose to 4 x 10^4 **resulted in mice that were completely protected from tumor establishment by treatment with antisense JF2S** (bolded data columns, Table 1). Treatment with the corresponding sense JF1S or scrambled JF14S control oligonucleotides was ineffective. In addition, while the nonspecific IgG MOPC 31C control was inactive, mAb 26-2F using one particular regimen was also highly effective in reducing tumor formation. Importantly, for both JF2S and mAb 26-2F treatment, mice that were tumor free at termination of treatment (day 49) remained so until sacrifice on day 70. Thus, this optimized model (i.e., 100% tumor take in controls coupled with appropriate therapeutic sensitivity at a cell injection dose of 4 x 10^4/mouse) will now be used routinely for therapy trials of antiAng antagonists and as a predictor of anti-metastatic activity in assays to be described in the next section.

Preclinical Mouse Model for Breast Cancer Metastasis. A major effort has been placed on the development of a model of metastasis which is both reproducible in the extent of metastasis present in untreated controls (ideally 100%) and conserving in the amount of time required to obtain meaningful, statistically significant results. As noted above, difficulties in completing this task were encountered during Year 1. This obstacle has now, we believe, been overcome. At the end of Year 1 experiments were underway to incorporate the modifications suggested by Dr. Price into the protocols for the MDA-MB-435 orthotopic metastasis model in order to obtain an assay system suitable for our purposes. These investigations indicated to us that although an anti-metastatic therapeutic effect was observed with an antiAng antagonist, the model still lacked reproducibility in obtaining 100% lung metastasis in controls.

Additionally, it still required an inordinately lengthy period of time to complete a single experiment. In an example of one such experiment (data shown in Tables 2 & 3), tumor cells were injected on day 0 followed by systemic treatment with Ang antisense JF2S, sense control JF1S or PBS beginning on day 1. Primary tumors in the mfp were removed after reaching a mean diameter of 14 mm which occurred beginning at approximately week 12 (removal is necessary in order for mice to survive for the time period necessary to develop metastases). Mice were sacrificed when they became distressed (as indicated by weight loss) or by day 149. As shown in Table 2, determining whether a complete therapeutic response (i.e., 100% protection from formation of metastasis) occurred as the result of treatment was not possible since only 4 of the 7 PBS-treated controls developed metastatic lesions, as assessed macroscopically. However, a comparison of lung weights (reflecting tumor load) among the groups revealed that treatment with JF2S was indeed therapeutically effective (Table 3). In the first data column of Table 3 average lung weights for all mice in the experiment treated with either PBS, control JF1S or antisense JF2S as a percentage of the value for the PBS-treated group (100%) are shown. While there was no apparent difference between JF1S- and PBS-treated mice, a 37% reduction in lung weight was observed for the JF2S-treated group, in spite of the fact that there was no difference in the size of the primary tumors among these groups. Importantly, the therapeutic effect of JF2S was still apparent when data obtained by further subdividing subsets of experimental mice were more closely scrutinized. Values in data column 2 are for only those mice whose primary tumor reached a diameter of 14 mm and were removed, while values shown in column 3 were obtained only from mice in the previous subset in which metastatic lung lesions could actually be observed. In this latter set, the 46% decrease in the average weight of lungs found in JF2S- versus PBS-treated mice is of statistical significance (one-tailed *t* test). **Thus, at this point we had demonstrated for the first time that the antagonization of Ang can lead to a substantial decrease in the formation of spontaneous metastases arising from a primary tumor growing in its natural setting.**

Although we considered this to be a major step forward, the lack of reproducible, i.e., 100% formation of metastatic lesions in control lungs coupled with the length of time needed to complete an experiment (~19 weeks), remained problematic.

At the 1998 meeting of the American Association for Cancer Research in March a poster by Dr. R. Mukhopadhyay, a member of Dr. Price's research group, presented data on a new variant of the MDA-MB-435 cell line, designated MDA-MB-435L2, which was selected for increased expression of the integrin $\alpha 6$. This new cell line was purported to produce a higher incidence of lung metastases when injected orthotopically into control athymic mice. Dr. Price kindly supplied us with these cells. We have recently completed an experiment in which these cells were orthotopically injected into the mfp of athymic mice followed by systemic treatment with antisense JF2S, sense control JF1S, mAb 26-2F or control PBS. At week 11, similar to what was observed with the parent cell line, a few mice in this current experiment carried primary tumors that had reached a diameter of 14 mm, the size at which according to protocol the tumor was to be removed. In the course of the initial surgeries the first two mice unexpectedly died. Autopsies revealed that death was due to metastasis which had surprisingly already infiltrated lung tissue. As a result, remaining mice were sacrificed the following week and examined for metastatic lung lesions. As shown in Table 4, *all* of the PBS control-treated mice had observable lung metastasis at sacrifice on days 77 to 84, a considerably shorter time period than we had observed using the parental line, where as shown above only 4 out 7 mice had developed metastases by an average sacrifice date of day 134. In this current experiment all of the sense control JF1S-treated mice also harbored lung metastasis. In contrast, only 6 out 8 and 4 out of 7 mice treated with mAb 26-2F or antisense JF2S, respectively, developed metastatic lesions in the lung. This was not due to a difference in the size of primary tumors since these average tumor sizes for those mice protected from lung metastases by the Ang antagonists were equivalent to that of PBS-treated mice. Thus, treatment

with mAb 26-2F or antisense JF2S was able to protect a subset of mice from developing lung metastasis in the presence of primary tumors sufficiently large to produce metastases in 100% of PBS- or sense control JF1S-treated mice. In Table 5 the efficacy of the Ang antagonists in reducing the extent of metastases in this experiment was also examined. In the first data column the average lung weights of all the mice in each group were compared to that of the PBS-treated group, which was set to 100%. Treatment with mAb 26-2F produced a slight decrease in average lung weight. More strikingly, while the average lung weight of sense control JF1S-treated mice was not different from that of PBS control mice, treatment with antisense JF2S resulted in a 40% drop in average weight compared with PBS treatment. To determine whether this positive effect was skewed by the presence of the metastasis-free mice in the JF2S group, we examined the average lung weights in only those mice who developed metastasis. The average lung weight of the 4 mice in the antisense-treated group was still 31% lower than that of the PBS-treated mice (data column 2). Therefore, not only was antisense JF2S able to *completely protect* a subset of mice from developing observable metastasis, but, as in the previous experiment discussed above, it produced a partial response in the remaining mice by decreasing the extent of metastasis as compared to controls.

The orthotopic metastasis model using MDA-MB-435L2 cells will now be used routinely in our therapeutic evaluations.

Drug Development.

i) *Chimerized antibody.* A report describing the construction and anti-breast tumor activity of the chimerized version of murine mAb 26-2F appeared during 1998 in the *Proceedings of the National Cancer Institute.* A reprint is included in the Appendix.

ii) *Humanized antibodies.* Since in some cases an immune response can still occur in patients directed against the mouse V region portions of chimeric antibodies (12), fully humanized versions of murine mAbs constructed by CDR grafting techniques are emerging for use clinically, although it is not certain at present that they will necessarily offer increased potency or decreased immunogenicity over chimeric antibodies in patients. Nevertheless, as part of our drug development program we will produce this product in addition to the chimeric antibody described above. Toward this end we have been collaborating with Dr. K. R. Acharya at the University of Bath, UK, an expert in x-ray crystallographic techniques and protein modeling. Dr. Acharya, in continuing collaboration with this Center has solved the crystal structures of both bovine (13) and human Ang (14) to 1.5 and 2.0 Å, respectively. As a step toward humanization, two batches of Fab fragments derived from mAb 26-2F have been prepared by us and supplied to Dr. Acharya. From these preparations three sets of crystals of the Fab-human Ang complex have been produced. Encouragingly, Dr. Acharya, working at the synchrotron at Daresbury, UK, has shown that the crystals do indeed diffract and he has collected data sets with resolution averaging 3.1 Å. Data accumulated thus far as they relate to molecular characterization of the Ang-Fab complex are summarized in Table 6. Molecular replacement studies have begun using the AMoRe program and the position of Ang within the Fab fragment is being determined. Contact regions within the Ang molecule have already been identified and include Trp 89 and a segment which encompasses residues 36-41. Encouragingly, these residues coincide exactly with those previously identified by our epitope mapping studies (9). However, refinement of the x-ray structure to that optimal for use in the design of humanized antibodies will require data acquisition at somewhat higher resolution. This should be accomplished within the next several months.

iii) *Small Molecule Inhibitors.* Previous studies by us have demonstrated that the ribonucleolytic activity of Ang, although extremely weak against conventional substrates, is nonetheless essential for the biological action of the protein. Therefore, molecules that are potent inhibitors of this activity might have considerable potential as anti-cancer agents. Dr. Robert Shapiro, a colleague at the Center and collaborator on this project, is spearheading efforts to develop small molecule inhibitors of the enzymatic

activity of Ang to be used by us therapeutically. Using an initial kinetic approach he has identified two lead inhibitory compounds, 5'-diphosphoadenosine 2'-monophosphate (ppA-2'-p) and its 2'-deoxyuridine derivative (dUppA-2'-p). Although neither ppA-2'-p nor dUppA-2'-p binds sufficiently tightly to Ang be useful as a drug, the interactions are strong enough to allow structural studies to be performed. Interactions between Ang and these identified inhibitors are being solved both in solution by NMR analysis (being performed by Dr. Shapiro's collaborator Dr. Feng Ni at the National Research Council of Canada) and in the crystal state (being performed by Dr. Acharya). Progress on both fronts is being made and it is estimated that within six months detailed conformational characterizations will be in hand. These data together with computer-aided analysis using Quanta (Molecular Simulations) software will then allow for rational design and synthesis of inhibitors strong enough (estimated Ki values of 1-10 nM at physiological pH) to be used in mouse experiments. Thus, we are on schedule to begin therapy trials with small molecule inhibitors during Years 3-4.

Specific Aim 2: Combined effects of antiAng agents and cytotoxic drugs

In our proposed Statement of Work we planned to investigate the combined effects of Ang antagonists with cytotoxic drugs commonly used in the treatment of breast cancer beginning in Year 2. For these studies we will investigate the effects of combination treatments in the orthotopic setting, since as discussed above we have already been able to achieve 100% protection of primary tumor establishment using an antiAng antagonist alone. Therefore, as a first step we set out to determine the appropriate doses of two drugs, doxorubicin and cyclophosphamide, which would affect the growth rate of MDA-MB-435L2 cells. Tumor cells were injected orthotopically into the mfp on day 0. Doxorubicin (10 mg/kg body weight), cyclophosphamide (150 mg/kg body weight) or normal saline as diluent control was given ip on day 48 when the tumors had an average volume of 258 mm^3. No difference was observed by day 60 in the growth rate of the tumors in the doxorubicin-treated mice in comparison with the growth rate of the tumors in the saline control mice. In subsequent experiments we will use different dose protocols to achieve an effective anti-tumor range for this drug. However, by day 60 tumor size in the cyclophosphamide-treated mice was only 29% of that observed in the saline-treated mice. Using this dose multimodal experiments are under way with JF2S to determine if the combined of treatments will be more efficacious than either drug alone in protecting mice from lung metastasis in the orthotopic model.

CONCLUSIONS

During the second year of Department of Defense support we have met the goals for this time period as defined in the Statement of Work. We have shown using a sc prophylactic model for the growth of primary breast cancer in athymic mice that antiAng antagonists are extremely effective in preventing the establishment of breast cancer cells injected subcutaneously. Remarkably, *complete protection* of primary tumors has been achieved by treatment with the Ang antisense phosphorothioate JF2S. More extensive dose-response studies will be undertaken to determine if the same level of success can be achieved with the other class of antiAng drug under investigation, namely monoclonal antibody 26-2F.

We believe that the major indication for antiAng therapy will be for the treatment and/or prevention of breast cancer metastasis, the form of disease that kills patients. As a result, a major effort has been placed on establishing in the laboratory a relevant metastatic model for evaluation of antiAng metastatic effects. It is becoming increasingly evident that models in which tumors are placed into their natural setting, i.e., in the mfp in the case of breast cancer, are more relevant for the study of tumor

biology and may be more predictive of the clinical response to anti-breast cancer drugs under development. At the time this grant application was written the only orthotopic model for breast cancer utilized the MDA-MB-435 cell line developed by Dr. Price. Under her guidance we attempted to adapt this assay for our purposes. We have now succeeded by using a new highly metastatic variant of the original cell line. The initial difficulties that we encountered in establishing a model that i) resulted in 100% formation of lung metastasis in control-treated mice, and ii) reduced the length of time to complete an experiment to less than the original 19 weeks, have now been overcome. Excitingly, in the first use of this model we were able to demonstrate for the first time *that antiAng antagonists potently interfered with the formation of lung metastasis derived from breast cancer cells implanted orthotopically into their natural setting.* This confirmed initial results of experiments using the parental cell line. We will now use this new model system for all future studies of metastasis.

We have also pursued our drug development program to the stage where construction of humanized versions of the murine neutralizing monoclonal antibody, 26-2F, can soon begin. (A chimerized antibody has already been prepared). Critical contact points between the Ang protein and the antigen-combining site of mAb 26-2F are being identified by x-ray crystallography. These determinations, nearing completion, will greatly facilitate the design of humanized counterparts of the mouse antibody. Additionally, progress is continuing to be made on the design of small molecule inhibitors of Ang. Structures of complexes between Ang and lead drug candidates are being investigated both in solution (using NMR) and in the crystal state (using x-ray crystallographic techniques). The data generated will be used in conjunction with state-of-the-art computer modeling to rationally design inhibitors of sufficient strength to be tested therapeutically during Years 3-4 as planned.

Finally, we have begun studies to evaluate the effects of combined treatments using Ang antagonists and more conventional chemotherapeutic drugs, as outlined in Specific Aim 2. Two drugs are under evaluation in regard to the proper doses to be used. We have already entered one of them, cyclophosphamide, into combination experiments with antiAng antisense JF2S in the orthotopic model of metastasis.

Thus, we continue to validate the proposition that the use of antagonists directed against Ang, either alone or in combination therapies, should be effective clinically for the prevention and/or treatment of metastatic breast cancer.

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STATEMENT OF WORK

The Statement of Work has been edited to reflect the development of Ang antisense reagents as anti-breast cancer therapeutics. We have substituted this class of drug, which in our preclinical models has shown great promise, for the Ang(108-123) synthetic peptide which was originally included to be used in our research plan - this latter peptide has not been tested as yet for efficacy against breast cancer. In the context of time and resources, we will therefore concentrate for the remainder of the granting period on the development of antisense drugs and immunoglobulin-based molecules (as proposed originally) for the treatment of breast cancer. The revised document follows on the next two pages with the only statement relevant to this change bolded.

Statement of Work (Revised)

Specific Aim 1: Antitumor effects of individual Ang antagonists

Task 1: Year 1, Months 1-2: Optimize orthotopic tumor model in athymic mice in regard to primary and metastatic growth of mfp-implanted MDA-435 and MCF-7 cells. (Mice=90).

Task 2: Year 1, Months 1-12: Prepare 4 batches of mouse ascites fluid for the production of mAb 26-2F to be used for therapy. (Mice=204).

Task 3: Year 1, Months 3-12: Test individual Ang antagonists for their capacity to interfere with the establishment, growth and metastatic spread of these two tumor types using the optimized orthotopic model. (Mice=704).

Task 4: Year 1, Months 3-12: Identify mechanism(s) by which tumor inhibition is occurring which involves histochemical staining analysis of blood vessel density and areas of necrosis and apoptosis in paraffin-embedded tumor tissue and by following concentrations of both human and mouse Ang from treated and control mouse serum.

Task 5: Year 1, Months 3-12: Determine whether any effective antiAng antagonists are cytotoxic to tumor cells *in vitro*.

End of Year 1: Identify lead antiAng compounds for clinical development; these are anticipated to be **antisense agent JF2S** and mAb 26-2F.

Task 6: Year 2, Months 1-4: Prepare a final 2 batches of mouse ascites for production of mAb 26-2F for therapy while chimeric antibody is being produced. (Mice=102).

Task 7: Year 2, Months 1-4: Prepare optimal stabilized Ang blocker, based on the structure of mAb 26-2F, as advised by our expert consultants in these respective fields; test these for therapeutic efficacy versus their parent compounds. (Mice=256).

Task 8: Years 3-4: Small molecular weight inhibitors that are being designed rationally on the basis of the 3-dimensional structure of Ang will be evaluated for their antitumor effects. (Mice=288).

Specific Aim 2: Combined effects of antiAng agents and cytotoxic drugs

Task 1: Year 2, Months 5-12: Test therapeutic regimens that combine the two optimized antiAng drug candidates with six cytotoxic treatments used clinically for the management of breast cancer in an efficacy "screening mode". (Mice=672).

End of Year 2: Identify those cytotoxic drugs that when combined with the two antiAng agents result in the most efficacious combination therapies.

Task 2: Year 3, Months 1-12: Examine rigorously the most promising combinations of antiAng and cytotoxic treatments identified during Year 2; confirm that they should be considered as candidates for clinical testing. (Mice=576).

Task 3: Year 3, Months 1-12: Perform mechanistic studies as above on the mode of tumor killing for the most efficacious combinatorial regimen.

Task 4: Year 3, Months 1-12: Perform *in vivo* toxicology studies on the most efficacious regimens. (Mice=336).

End of Year 3: Candidate combined therapies for potential clinical evaluation will be identified; safety in animals will be certified.

Specific Aim 3: Effect of addition of other antiangiogenic compounds to successful regimens.

Task 1: Year 4, Months 1-12: Evaluate the potency of three other antiangiogenic compounds separately and in combination with i) two previously identified potent cytotoxic treatments and ii) the same two cytotoxic treatments together with the two antiAng drug candidates. (Mice=576).

End of Year 4: Identify combination treatments that are potentially more effective than any of those previously examined that warrant future preclinical development.

APPENDICES (illustrations, reprint)

Pages 16-22 & reprint

Table 1. Prevention of MDA-MB-435 tumor growth in athymic mice by treatment with Ang neutralizing mAb 26-2F or Ang antisense JF2S[a]

	Cell Number					
Treatment	12.5[b]	6.25	4	4	2	2
PBS	0/11[c] (0%)	0/9 (0%)	**0/8 (0%)**	**0/8 (0%)**	2/8 (25%)	3/8 (38%)
MOPC 31C (control)			**0/7 (0%)**	**0/8 (0%)**		
mAb 26-2F	3/8 (38%)	4/8 (50%)	**4/8 (50%)**	**3/8 (38%)**	7/8 (88%)	7/8 (88%)
Sense, JF1S (control)			**0/8 (0%)**	**0/8 (0%)**		
Antisense, JF2S	5/10 (50%)	5/9 (56%)	**8/8 (100%)**	**8/8 (100%)**	6/8 (75%)	7/7 (100%)
Scrambled, JF14S (control)				**0/14 (0%)**		

[a] Mixtures of cells (at the cell dose/mouse indicated) with either PBS, nonspecific IgG control MOPC 31C (240 μg/mouse), mAb 26-2F (240 μg/mouse), Ang sense control JF1S (400 μg/mouse), Ang antisense JF2S (400 μg/mouse) or Ang scrambled control JF14S (400 μg/mouse) were injected into the surgically exposed mfp on day 0. Mice were then treated sc with one half the day 0 dose of the same material 6 times per week for the first two weeks, followed by the same dose given 4 times per week until day 49. [b] Number of MDA-MB-435 cells (x 10^{-4}) injected per mouse. [c] Number of tumor-free mice on day 49/total number of mice; expressed as a percentage in parentheses below the fraction.

Table 2. Treatment of MDA-MB-435 lung metastasis with Ang antisense JF2S: Prevention of formation of metastases [a]

	Incidence of metastasis
PBS	4/7 [b] (57%)
Sense control, JF1S	5/8 (62%)
Antisense, JF2S	4/8 (50%)

[a] MDA-MB-435 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (4×10^6/mouse). The next day treatment is begun with either PBS (diluent control), Ang sense control JF1S (300 μg/mouse) or Ang antisense JF2S (300 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice. [b] Number of mice in which macroscopically observable metastasis was observed in the lungs at sacrifice/total number of mice; expressed as a percentage in parentheses below the fraction.

Table 3. Treatment of MDA-MB-435 lung metastasis with Ang antisense JF2S: Reduction of extent of metastases [a]

	All mice	All mice with primary tumor removed [b]	Mice with observable lung metastasis [c]
PBS	100 [d] (7)	100 (6)	100 (4)
Sense control, JF1S	111 (8)	137 (5)	105 (5)
Antisense, JF2S	63 (8)	59 (6)	54 [e] (4)

[a] MDA-MB-435 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (4×10^6/mouse). The next day treatment is begun with either PBS (diluent control), Ang sense control JF1S (300 μg/mouse) or Ang antisense JF2S (300 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice. [b] Mice whose primary tumor was removed after reaching a mean diameter of at least 14 mm. [c] Mice in which macroscopically observable metastasis was observed in the lungs at sacrifice. [d] Average lung weight in percent of the indicated group in comparison with the PBS control, which is set at 100. The number of mice in the group is listed below in parentheses. [e] By one-tailed *t* test $p < 0.05$ for the difference between the average lung weights of the antisense JF2S- and PBS-treated groups.

Table 4. Treatment of MDA-MB-435L2 lung metastasis with Ang neutralizing mAb 26-2F or Ang antisense JF2S: Prevention of formation of metastases [a]

	Incidence of metastasis
PBS	8/8 [b] (100%)
mAb 26-2F	6/8 (75%)
Sense control, JF1S	8/8 (100%)
Antisense, JF2S	4/7 (57%)

[a] MDA-MB-435L2 breast tumor cells, more aggressive metastatic variants of the original MDA-MB-435 cells, are injected into the surgically exposed mfp of athymic mice on day 0 (4×10^6 /mouse). The next day treatment is begun with either PBS (diluent control), mAb 26-2F (120 μg/mouse), Ang sense control JF1S (300 μg/mouse) or Ang antisense JF2S (300 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice. [b] Number of mice in which macroscopically observable metastasis was observed in the lungs at sacrifice/total number of mice; expressed as a percentage in parentheses below the fraction.

Table 5. Treatment of MDA-MB-435L2 lung metastasis with Ang neutralizing mAb 26-2F or Ang antisense JF2S: Reduction of extent of metastases [a]

	All mice	Mice with observable lung metastasis [b]
PBS	100 [c] (8)	100 (8)
mAb 26-2F	82 (8)	93 (6)
Sense control, JF1S	109 (8)	109 (8)
Antisense, JF2S	60 [d] (7)	69 [e] (4)

[a] MDA-MB-435L2 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (4 x 10^6/mouse). The next day treatment is begun with either PBS (diluent control), mAb 26-2F (120 μg/mouse), Ang sense control JF1S (300 μg/mouse) or Ang antisense JF2S (300 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice. [b] Mice in which macroscopically observable metastasis was observed in the lungs at sacrifice. [c] Average lung weight in percent of the indicated group in comparison with the PBS control, which is set at 100. The number of mice in the group is listed below in parentheses. [d] By one-tailed t test $p < 0.0005$ for the difference in the average lung weights of the antisense JF2S- and PBS-treated groups; $p < 0.005$ for the difference in the average lung weights of the antisense JF2S- and sense JF1S-treated groups. [e] By one-tailed t test, $p < 0.005$ for the differences in average lung weights between either the PBS- or sense JF1S-treated control groups and the antisense JF2S-treated group.

Table 6. Data processing statistics

	Prep. 1, Expt. 1	Prep. 1, Expt. 1	Prep. 2, Expt. 2
Space group	C2	C2	C2
Cell dimensions	117.08 X 81.74 X 87.42 Å β=110.06°	120.96 X 72.80 X 87.81 Å, β=112.86°	120.64 X 72.33 X 87.39 Å, β=112.83°
Resolution (Å)	40-3.2	40-3.0	40-2.8
N_m §	49700	35736	63733
N_u ‡	11440	11248	14611
R_{merge}, %	11.7 (42.7)[1]	10.1 (34.9)[2]	10.9 (49.9)[3]
Completeness, %	85.9 (47.2)	80.1 (76.3)	86.1 (65.1)
<I/σ(I)>	8.9 (1.5)	5.8 (1.5)	5.5 (1.2)

§ Number of measurements.
‡ Number of unique reflections.
[1] Last resolution shell 3.31-3.20 Å.
[2] 3.09-3.00 Å.
[3] 2.88-2.80 Å.

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Chimeric anti-angiogenin antibody cAb 26–2F inhibits the formation of human breast cancer xenografts in athymic mice

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ABSTRACT **Angiogenin (Ang), an inducer of neovascularization, is secreted by several types of human tumor cells and appears critical for their growth. The murine anti-Ang monoclonal antibody (mAb) 26–2F neutralizes the activities of Ang and dramatically prevents the establishment and metastatic dissemination of human tumor cell xenografts in athymic mice. However, for use clinically, the well-documented problem of the human anti-globulin antibody response known to occur with murine antibodies requires resolution. As a result, chimeric as well as totally humanized antibodies are currently being evaluated as therapeutic agents for the treatment of several pathological conditions, including malignancy. Therefore, we have constructed a chimeric mouse/human antibody based on the structure of mAb 26–2F. Complementary DNAs from the light and heavy chain variable regions of mAb 26–2F were cloned, sequenced, and genetically engineered by PCR for subcloning into expression vectors that contain human constant region sequences. Transfection of these vectors into nonproducing mouse myeloma cells resulted in the secretion of fully assembled tetrameric molecules. The chimeric antibody (cAb 26–2F) binds to Ang and inhibits its ribonucleolytic and angiogenic activities as potently as mAb 26–2F. Furthermore, the capacities of cAb 26–2F and its murine counterpart to suppress the formation of human breast cancer tumors in athymic mice are indistinguishable. Thus cAb 26–2F, with its retained neutralization capability and likely decreased immunogenicity, may be of use clinically for the treatment of human cancer and related disorders where pathological angiogenesis is a component.**

Angiogenesis, a multifaceted process by which new blood vessels form, occurs in many physiological and pathological situations, including cancer. Indeed, the critical contribution of angiogenesis to the growth, invasiveness, and metastatic dissemination of tumor cells is now well documented (reviewed in refs. 1 and 2). Mediators that affect angiogenesis are thus appropriate molecular targets against which to direct anticancer therapeutic strategies. One of these, angiogenin (Ang), a unique member of the ribonuclease superfamily of proteins, is a potent inducer of neovascularization and is serving as the focus of ongoing investigations into its structure/function relationships and clinical applications (reviewed in ref. 3).

Because Ang was originally isolated from medium conditioned by a human tumor cell line (4) and subsequently shown to be expressed by several histologically distinct types of human tumors (5), inhibitors of its functions have been developed to evaluate their antitumor effects. One of these, the murine monoclonal antibody (mAb) 26–2F, neutralizes the ribonucleolytic, angiogenic, and mitogenic activities of human Ang (6, 7). It is an IgG1κ with a binding affinity of 1.6 nM that recognizes a discontinuous epitope in Ang involving Trp-89 and residues in the segment 38–41, located in two adjacent loops of the Ang 3-dimensional structure (6, 8). Although not directly cytotoxic to tumor cells *in vitro*, mAb 26–2F is extremely effective in interfering with their establishment and metastatic spread in athymic mice (9–11). Thus, Ang antagonists should be of major clinical utility for the treatment of cancer.

The use of murine antibodies in patients is problematic, owing to their decreased serum half-lives and induction of human anti-mouse antibody immune responses, directed mainly against mouse Ig constant (C) regions (12–15). The latter is of particular concern in the case of antiangiogenesis therapies, where chronic administration of therapeutic agents may be required. To minimize this problem, chimeric antibodies have been genetically engineered in which murine heavy (H) and light (L) chain variable (V) domains are combined with human C regions, thereby replacing ≈70% of the murine antibody molecule with human sequences (16, 17). Several of these chimerized antibodies are under evaluation in patients for a variety of diseases (18–20). Therefore, as a first step toward producing an anti-Ang antibody amenable to clinical testing, a mouse/human chimeric analogue of mAb 26–2F has been constructed. Here we describe the cloning and sequencing of the V_L and V_H domains of mAb 26–2F and their expression together with human C regions as a fully assembled chimeric mAb (cAb 26–2F). cAb 26–2F is very similar if not identical to its murine counterpart in binding affinity, Ang neutralization capacity, and, importantly, in its antitumor activity against human breast cancer xenografts in athymic mice.

MATERIALS AND METHODS

Mice. Female athymic mice were obtained at 5 weeks of age from the isolator bred colony of Charles River Breeding Laboratories and maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment. Experiments were begun 1 week later.

Monoclonal Igs. The mAb 26–2F (6) was purified from ascites fluid by affinity chromatography using GammaBind Plus Sepharose (Pharmacia). Ascites fluid (80 ml) was diluted 1:1 with PBS, centrifuged, and the supernatant filtered through a glass fiber filter and 0.2 μm cellulose nitrate filter. After a

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Abbreviations: Ang, angiogenin; cAb, chimeric antibody; CAM, chorioallantoic membrane; H and L, Ig heavy and light chains, respectively; V and C, Ig variable and constant regions, respectively.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF039414 (V_L) and AF039415 (V_H)].

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further dilution with PBS to 400 ml, the antibodies were adsorbed onto the gel, washed with PBS, and eluted with 0.1 M glycine·HCl into tubes containing an appropriate amount of 1 M Tris·HCl for neutralization. Following dialysis against 0.9% NaCl, the antibodies were quantified by enzyme-linked immunoadsorbent assay (ELISA), and stored at −70°C. The chimerized analogue of mAb 26–2F (cAb 26–2F, see below) obtained from each of the transfectoma cell types was purified from ascites fluid as described above. MOPC 31C, a nonspecific IgG1κ-secreting mouse hybridoma (CCL 130, American Type Culture Collection) was propagated, and IgG purified from ascites as described (9).

Cell Lines. The murine nonproducing myeloma cell lines P3X63-Ag8.653 (P3X) (CRL 1580) and Sp2/0 (CRL 1581) were obtained from the American Type Culture Collection. The estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-435 human breast cancer cell lines were supplied by Marc E. Lippman (Georgetown University Medical Center) and Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center), respectively. We have determined that both cell lines secrete Ang *in vitro*. All cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and antibiotics (growth medium).

Isolation of mAb 26–2F V_L and V_H Region cDNAs. Polyadenylated RNA was prepared from mAb 26–2F-producing hybridoma cells using the PoliATtract System 1000 mRNA isolation kit (Promega). The procedure followed for V_L and V_H cDNA isolation was essentially that of Coloma *et al.* (21) with minor modifications. For V_H first-strand cDNA synthesis, the reaction used the C_H1 antisense primer MγC.C_H1 AS and avian myeloblastosis virus reverse transcriptase (Promega). V_H cDNA amplification was performed using MγC.C_H1 AS as the antisense primer and a set of three universal sense primers complementary to the N termini of most V_H leader sequences (MHALT1.RV, MHALT2.RV, and MHALT3.RV). The V_L domain-encoding cDNA was obtained using the Pharmacia Mouse ScFv Module/Recombinant Phage Antibody system. V_L cDNA amplification was performed using *Taq* DNA polymerase (Promega), the C region MC_k AS.XBA antisense primer, and five universal sense primers complementary to the N terminus of V_L leader sequences (MLALT1.RV, MLALT2.RV, MLALT3.RV, MLALT4.RV, and MLALT.5).

PCR amplifications of both V_H and V_L cDNAs were carried out for 30 cycles in a MicroCycler thermal controller (Eppendorf) under the following conditions: 1 min denaturing (94°C), 2 min annealing (55°C), 2 min extension (72°C) followed by a final extension step of 7 min (72°C). The products were analyzed by electrophoresis in a 1.5% TAE agarose gel stained with ethidium bromide. The amplified cDNAs were then electrophoresed on a 2% low melting agarose gel in 0.5× TAE and eluted using a Magic PCR Preps DNA Purification kit (Promega).

Subcloning and Sequencing. Each V domain-encoding cDNA was ligated into a pT7Blue T vector (Novagen) using T4–DNA ligase (Promega). The ligation mixture was used for transformation of NovaBlue competent cells (Novagen). Plasmid DNA minipreps were analyzed by 1.5% agarose gel electrophoresis after digestion with appropriate restriction enzymes. Several clones containing inserts of the expected size were sequenced in both directions using a Sequenase 2.0 sequencing kit (United States Biochemicals).

V Domain cDNA Engineering. To clone V_L and V_H cDNAs into their appropriate expression vectors, they were each subjected to further PCR reactions using the following primers: H chain sense primer: MHALT2.RV (21) hybridizing to the N terminus of the H chain leader sequence and containing the *Eco*RV restriction site for cloning into the H chain expression vector. H chain antisense primer (H-P2 antisense): CTAGCTAGCTGAGGAGACGGTGACTGAGGTTCCT hybridizing to the J region and containing a *Nhe*I site for cloning into the C_H1 region of the H chain expression vector. L chain sense primer (L-P2 sense): GGGGATATCCACCATGGAGACAGACACACTCCTGCTATGGGTCCTGCT corresponding to oligonucleotide MLALT1.RV (21), containing a 10 nucleotide extension at the 3′ end and hybridizing to the N terminus of the L chain leader sequence. An *Eco*RV site is present for cloning in the L chain expression vector. L chain antisense primer (L-P2 antisense): AGCCGTCGACTTACGTTTCAGCTCCAGCTTGGTCCCAG hybridizing to the J region and containing a splicing signal sequence as well as a *Sal*I site for cloning into the intronic sequence of the L chain expression vector.

The amplified products were gel purified and cloned into pT7Blue T vectors for sequencing as described above. For both modified V_L and V_H domains, the cDNAs from two identical clones were excised with either *Eco*RV and *Sal*I (for V_L) or with *Eco*RV and *Nhe*I (for V_H) for cloning into expression vectors.

Construction of Chimeric Genes. The L and H chain expression vectors (pAG4622 and pAH4604, respectively) were constructed (21) and kindly provided by Sherie L. Morrison (University of California, Los Angeles). The pAG4622 vector contains the genomic sequence encoding the C-region domain of the human κ L chain and the *gpt* (22) selectable marker. The pAH4604 vector contains the *his*D (23) selectable marker in addition to sequences encoding the human H chain γ1 C-region domain. The promoter region in each vector is derived from the anti-dansyl mAb 27–44 (21). For each V_L and V_H domain, cDNA fragments obtained from two identical clones were appropriately digested and ligated into their respective expression vectors. The ligated products were used to transform HB101 competent cells (Promega) and the recombinant vectors were isolated using the Wizard Plus Maxipreps DNA purification system (Promega). Prior to transfection, they were linearized with the *Pvu*I isoschizomer BspCI restriction enzyme (Stratagene) and gel purified.

Transfection and Selection. The chimeric H and L chain expression plasmids were cotransfected into SP2/0 or P3X nonproducing myeloma cells by electroporation as described (21). Following transfection, the cells were kept on ice for 10 min, diluted in growth medium, and placed into 96-well tissue culture plates (1×10^4 cells per well). The cells were refed 48 hr later with growth medium containing histidinol (Sigma) at a final concentration of 5 or 10 mM for SP2/0 or P3X cells, respectively. After ≈14 days, supernatants from growing colonies were screened by ELISA for the presence of chimeric antibodies.

Two selected stable transfectants, P4–5 and S13–1, obtained from the transfection of P3X or SP2/0 cells, respectively, were subcloned twice by limiting dilution. To obtain sufficient material for further analysis, cAb 26–2F from each cell source was purified from ascites fluid as described above.

Immunoassays. *Screening ELISA.* Chimeric antibody producing transfectomas were detected by a modification of the screening ELISA described in Fett *et al.* (6). Briefly, affinity-purified goat anti-human IgG Fc (γ-chain specific) and goat anti-human κ chain (each at 10 μg/ml, Organon Teknika–Cappel), or human Ang (1 μg/ml) was coated onto 96-well plates. Following blocking of the wells with 0.5% ovalbumin, 50 μl of culture supernatant diluted 1:1 with 0.25% ovalbumin was added. After a 2-hr incubation at room temperature, the plates were washed and alkaline phosphatase-labeled goat anti-human IgG (Kirkegaard & Perry Laboratories) was added to each well, followed 1 hr later by addition of *p*-nitrophenyl phosphate to the washed plates. The reaction was stopped with 3 N NaOH and absorptivities were measured on a Dynatech MR600 ELISA plate reader at 405 nm with a turbidity reference of 630 nm.

Radioimmunoassay for binding affinity. A competition radioimmunoassay for binding affinity (6) with the following

modifications was used to determine IC_{50}s, the concentration of unlabeled Ang at which the binding of its iodinated derivative is decreased by 50%. Plates were coated (10 μg/ml in borate coating buffer, 50 μl per well) with either goat anti-mouse IgG Fc (γ-chain specific, Organon Teknika–Cappel) for capture of mAb 26–2F or goat anti-human IgG Fc (see above) for capture of the chimeric antibody. Radioactivity was determined using a Micromedic 4/600plus Gamma Counter.

Concentration Determinations. Ig concentrations were determined spectroscopically, assuming that a 1 mg/ml solution has an absorbance of 1.43 at 280 nm.

tRNA Assay. Formation of perchloric acid soluble fragments from yeast tRNA was measured as described (24).

Angiogenesis Assay. The chicken chorioallantoic membrane (CAM) assay was used according to Fett *et al.* (6).

Western Blot Analysis. The general procedures for SDS/10% PAGE, transfer, and Western blotting have been described (25). Samples were boiled in a buffer containing 5% 2-mercaptoethanol before loading onto the gel. For detection of human components, goat anti-human IgG Fc and κ chain antibodies were used. Ig chains were visualized with alkaline phosphatase-labeled rabbit anti-goat IgG and nitroblue tetrazolium as substrate.

Antitumor Activity *in Vitro*. Direct cytotoxicity of cAb 26–2F toward MDA-MB-435 and MCF-7 cells was examined using a described [^{3}H]thymidine assay (9).

Antitumor Activity *in Vivo*. This was assessed by a modification of the orthotopic model of human breast cancer tumor growth in athymic mice described by Price *et al.* (26). Tumor cells (MDA-MB-435 or MCF-7) were harvested by standard trypsinization procedures, washed in Hanks' buffered salt solution, and counted by trypan blue exclusion hemacytometry. Viable cells (MDA-MB-435, 5×10^5 in 10 μl, or MCF-7, 1×10^6 in 20 μl) were injected into the surgically exposed mammary fat pad using a manual repeating dispenser (Hamilton). For MCF-7 cells a pellet of 17β-estradiol (0.72 mg per pellet, 60-day release; Innovative Research of America) was placed 1 cm from the site of tumor cell injection as the source of standard estrogen supplementation. The incision was closed with an autoclip and local subcutaneous treatment was begun within 30 min as described in the legend to Fig. 4. Tumor growth was monitored by caliper measurements.

RESULTS AND DISCUSSION

Isolation of cDNAs Encoding mAb 26–2F V Domains. Polyadenylated RNA was isolated from mAb 26–2F-producing hybridoma cells. cDNA sequences encoding the mAb 26–2F variable domains (V_L and V_H) were amplified by PCR using gene-specific primers designed to hybridize to the leader sequence of each domain (5′ primers) and to the C region N-terminal coding sequences positioned immediately downstream of the V–J region (3′ primers). Using this strategy, no amino acid substitutions that could effect chimeric antibody activity are introduced into the V_L or V_H cDNAs.

V_L and V_H amplified cDNAs were then cloned into pT7Blue T vectors and recombinant plasmids, isolated from independent clones, were sequenced. For each type of cDNA, at least two clones were identical. The nucleotide and deduced amino acid sequences for the V_L and V_H domains of mAb 26–2F are shown in Fig. 1. According to the classification of Kabat *et al.* (27), the DNA sequences encode V_H IIID and V_k III V regions, each including three complementarity-determining regions and four framework regions. The deduced amino acid sequence of the first 16 N-terminal amino acids of each V domain is identical to that obtained by Edman degradation of the protein (data not shown).

Construction and Expression of Chimeric Antibody Genes. V_H and V_L cDNAs were modified at their 3′ end by removing the N-terminal sequence of the murine C region and adding a

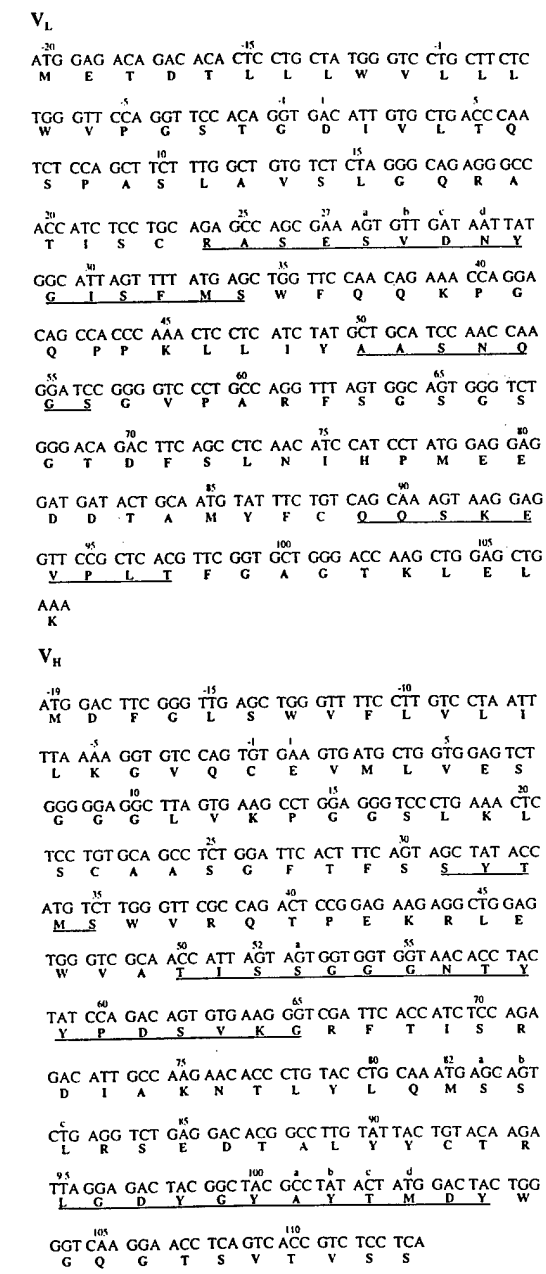

FIG. 1. Nucleotide and deduced amino acid sequences for mAb 26–2F V_L (*Upper*) and V_H (*Lower*) domains. The sequences were interpreted according to Kabat *et al.* (27). Underlined amino acids comprise the three complementarity-determining regions. Portions of the leader sequence are not necessarily correct because they correspond to the PCR primers.

splicing signal sequence at the V_L 3′ end. The resulting V_H and V_L cDNA-containing plasmids, prepared from independent clones, were digested with *Eco*RV and *Xba*I, gel purified, and amplified by PCR using primers complementary to the signal peptides (sense primers) and to the 3′ end (antisense primers) of each V_H and V_L domain. The gel-purified PCR products were cloned into pT7Blue T vectors and independent clones were sequenced. The sequence analyses confirmed that the expected DNA assembly had been achieved. For each modified V_H and V_L domain, the cDNA from two identical independent clones were excised with either *Eco*RV and *Sal*I (for V_L) or *Eco*RV and *Nhe*I (for V_H) and gel purified. The V_L and V_H cDNA products were ligated into their respective expression vectors. Several clones, isolated from HB101 competent cell transformation, were analyzed with appropriate restriction

enzymes. Recombinant vectors were isolated in duplicate from two distinct clones, each of which derived from independent V_L- or V_H-containing plasmid clones. Prior to transfection, the recombinant vectors were linearized with *Pvu*I and gel purified.

Combinations of chimeric H and L chain-containing vectors were cotransfected into either P3X or SP2/0 cells by electroporation. Cells were grown in 96-well plates and selected for the presence of the *his*D marker by including histidinol in the growth medium. Transfection efficiencies for both cell lines under these conditions were greater than 1 in 10^4. At approximately 2 weeks after transfection supernatants from surviving cells were assayed by ELISA. These indicated that the vast majority of transfectomas produced human Ig chimeras that bound to Ang; cells secreting only chimeric L chain genes were detected in a small percentage of wells. Two chimeric antibody producing master wells designated S13–1 and P4–5, obtained from the transfection of SP2/0 and P3X cells, respectively, were selected as stable transfectants and subcloned twice by limiting dilution.

Purification and Structural Characterization of cAb 26–2F. S13–1 or P4–5 transfectoma cells were injected into pristane-primed athymic mice to generate ascites fluid. Antibody was then subsequently isolated by protein G-Sepharose affinity chromatography. The total yield of purified cAb 26–2F from either transfectoma source was ≈3 mg per mouse.

Purified S13–1- and P4–5-derived chimeric antibodies were first subjected to 10 cycles of Edman sequence analysis. L and H chain N-terminal amino acids of both chimeric antibodies were identical (data not shown) and correspond to those of the original mAb 26–2F.

Western blot analysis using reagents specific for human κ and γ1 C region determinants showed that cAb 26–2F from either transfectoma cell source contained reduced chimeric L and H chains of the expected molecular weights (≈25,000 and 55,000, respectively) (Fig. 2). Under nonreducing conditions, cAb 26–2F derived from either S13–1 or P4–5 migrated to a position corresponding to 160,000 daltons (data not shown), thus indicating that the chimeric L and H chains were correctly assembled into complete H_2L_2 molecules.

The IC_{50}s for S13–1- and P4–5-derived cAb 26–2F are 2.1×10^{-9} M and 2.4×10^{-9} M, respectively, values that are essentially indistinguishable, within the error of the assay, to that obtained for mAb 26–2F (1.6×10^{-9} M).

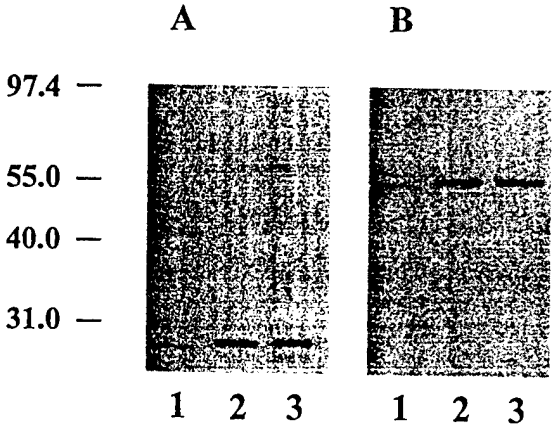

FIG. 2. Western blot analysis of cAb 26–2F. Reduced proteins (400 ng) were separated by SDS/10% PAGE and transferred to nitrocellulose sheets. These were incubated with either goat anti-human κ chain (*A*) or goat anti-human IgG Fc-specific (*B*) antibodies followed by treatment with alkaline phosphatase-labeled rabbit anti-goat IgG and nitroblue tetrazolium. Lane 1, mAb 26–2F; lane 2, cAb 26–2F from S13–1; lane 3, cAb 26–2F from P4–5. Molecular weight standards ($\times 10^{-3}$) are at left.

Table 1. Effect of cAb 26-2F derived from S13-1 or P4-5 myeloma cells on the activity of human Ang in the CAM assay

	mAb				MOPC	Assay		
Group	Ang	26-2F	S13-1	P4-5	31C	results*	$P^\dagger$	Status
I	+	−	−	−	−	25/45 (56)	0.0009	Active
II	+	+	−	−	−	10/45 (22)	0.9556	Inactive
III	+	−	+	−	−	11/46 (24)	0.8038	Inactive
IV	+	−	−	+	−	11/45 (24)	0.7594	Inactive
V	+	−	−	−	+	26/45 (58)	0.0004	Active
VI	−	+	−	−	−	9/42 (21)	0.9718	Inactive
VII	−	−	+	−	−	7/45 (16)	0.4492	Inactive
VIII	−	−	−	+	−	13/42 (31)	0.3258	Inactive
IX	−	−	−	−	+	15/45 (33)	0.2154	Inactive

Combined data represent three sets of assays. Each individual assay employed between 15 and 19 eggs. Amount applied per egg was 10 ng of Ang and 100 ng of IgGs.

*Results are expressed as the ratio of positive to total surviving eggs; the percentage of positive eggs is given in parentheses.

†Significance was calculated from χ^2 values of data recorded at 48 ± 2 hr based on comparison with water controls tested simultaneously (10 positive eggs/46 total surviving eggs, 22% positive). To be designated active samples must have a value of $P < 0.05$.

Functional Characterization of cAb 26–2F. A comparison of the capacity of cAb 26–2F with its murine counterpart to inhibit the angiogenic activity of Ang on the CAM is shown in Table 1. Statistical analysis by the χ^2 test indicates that cAb 26–2F purified from either S13–1 (group III, $P = 0.8038$) or P4–5 (group IV, $P = 0.7594$) is as potent as mAb 26–2F (group II, $P = 0.9556$) in inhibiting the biologic activity of an equimolar amount of Ang, which alone is highly active (group I, $P = 0.0009$). The control MOPC 31C is not inhibitory (group V, $P = 0.0004$). The Igs alone are inactive on the CAM (groups VI–IX, $P > 0.05$).

To this point, the structural and functional data taken together indicated that, as expected, the chimeric antibodies derived from transfection of either SP2/0 or P3X myeloma cells were identical. However, in the course of these studies it was observed that cells derived from clone S13–1 proliferated at a greater rate and, in general, maintained a higher percentage of viable cells in culture. In addition, S13–1 adapted easily to growth in protein-free medium, whereas P4–5 cells died under these conditions, an important consideration when large-scale production necessary for clinical trials is contemplated. For the above reasons, the remaining data to be

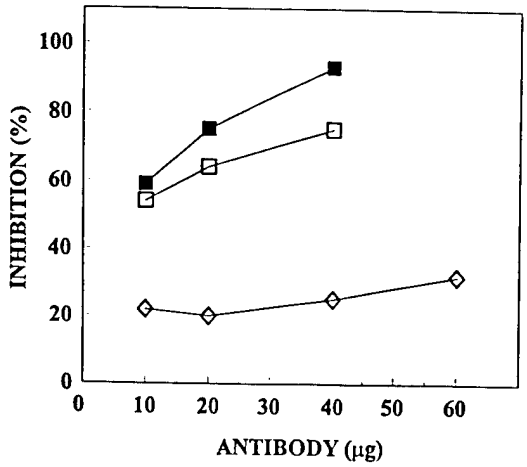

FIG. 3. Inhibition of the ribonucleolytic activity of Ang by mAb 26–2F (■), cAb 26–2F (□), or control MOPC 31C (◇). Ang was preincubated with the indicated amounts of Igs and assays were performed in 33 mM Hepes/33 mM NaCl, pH 6.8, at 37°C according to Shapiro *et al.* (24).

reported were generated with cAb 26–2F derived solely from clone S13–1, which will serve as the future source of the chimeric antibody.

The capacity of cAb 26–2F to inhibit tRNA degradation by Ang was determined by measuring the rate of formation of perchloric acid-soluble fragments. The inhibition curves obtained with mAb 26–2F, cAb 26–2F, and the control MOPC 31C are shown in Fig. 3. At 10 μg, the two antibodies are equally inhibitory, whereas at higher concentrations cAb 26–2F is only slightly less active.

The antitumor activity of cAb 26–2F was subsequently examined using modifications of an orthotopic tumor cell model (26). The results depicted in Fig. 4 indicate that cAb 26–2F is as effective as mAb 26–2F in preventing the formation of tumors of human breast cancer origin. Whereas all PBS- and control MOPC 31C-treated mice develop MDA-MB-435 (Fig. 4*A*) or MCF-7 (Fig. 4*B*) tumors by days 17 and 28, respectively, the chimeric and murine antibodies completely prevent the appearance of tumors in ≈40% (MDA-MB-435) and ≈50% (MCF-7) of the treated mice. Because cAb 26–2F does not interfere with thymidine uptake and, by inference, killing of tumor cells *in vitro* (data not shown), the antitumor effects observed most likely result from the inactivation of tumor-secreted Ang and subsequent interruption of the angiogenic process.

In summary, we have constructed a recombinant chimeric mouse/human anti-Ang antibody, cAb 26–2F, in which the V_L and V_H regions of mAb 26–2F were inserted into expression vectors containing C regions of human κ chains and γ1 H chains. The resultant chimera retains the properties of the original mAb, including potent activity against human tumor cell xenografts. As a consequence, cAb 26–2F should provide a powerful immunotherapeutic for the treatment of human cancer and other conditions where inhibition of pathological angiogenesis is desired.

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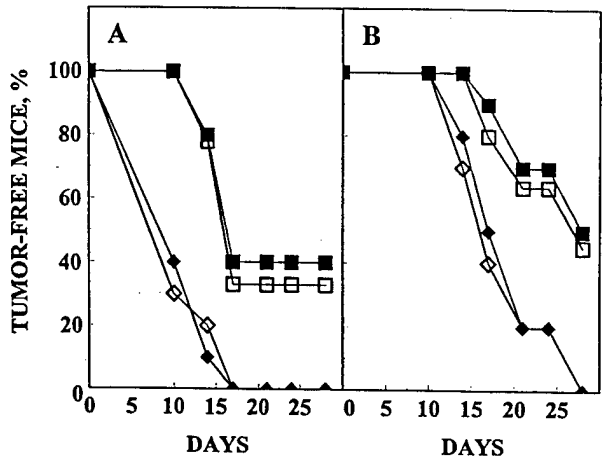

FIG. 4. Prevention of MDA-MB-435 (*A*) and MCF-7 (*B*) tumor formation by mAb 26–2F or cAb 26–2F. Tumor cells [5×10^5 (*A*) or 1×10^6 (*B*) per mouse] were injected into the surgically exposed mammary fat pad on day 0. For MCF-7 cells, a 17β-estradiol pellet was implanted in each mouse as a source of exogenous estrogen. Within 30 min of tumor cell injection the mice were treated with local subcutaneous injections of either PBS (♦) or Igs [mAb 26–2F (■), cAb 26–2F (□), MOPC 31C (◇); 240 μg/dose (*A*) and (*B*)]. Mice were then treated locally with 120 μg/dose (*A*) or 240 μg/dose (*B*) 6 times per week until sacrifice on day 28. $n = 10$ for all groups.

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